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AUTHOR(S):

Ikai, Atsushi

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# Mechanical Unfolding of a Knotted Protein Studied by Atomic Force Microscopy

Atsushi Ikai, Innovation Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta,  
Midori-ku, Yokohama, 226-8501, Japan <sup>1</sup>

**Abstract :** Carbonic dehydratase is a well known example of knotted proteins so that, if the C- and N-termini are pulled to opposite directions by a nano-mechanical means, the extended polypeptide chain would be left with a trefoil knot. Stretching of a nano-meter sized single protein molecule was accomplished by using an atomic force microscope and the result verified the mechanical tightening of the molecular structure due to the presence of the original knot, *i.e.*, the chain extension process was encountered by a strong resistance after about 20 nm of the chain was unfolded. When the same protein was genetically engineered so that no knot tightening would take place when it was stretched the protein was extended with less resistance. Knot in the case of carbonic dehydratase was shown to finalize the folding process of the protein from the denatured state.

## 1 Introduction

Knot forming folding patterns in protein conformation were once found for only a small number of proteins and considered to be rather exceptional aberrations. In recent years, however, many more cases of such folding patterns have been found and attracting keener attention of researchers with widely varying interests [1, 2, 3]. We have undertaken a single molecule stretching experiment on the well known case of carbonic dehydratase with a knot forming folding pattern at its C-terminal end. It is a protein of linearly polymerized 259 amino acid residues without disulfide crosslinks [4]. A simplified folding pattern of one of its variants, carbonic dehydratase II, is presented in Figure 2.21 [4]. The C-terminal chain element crosses at least three times with the rest of the chain, over, under and over, respectively, so that, if the protein is mechanically stretched from N- and C-terminal ends to opposite directions a trefoil knot would remain somewhere along the chain. Moreover, in the beginning of pulling from the two ends, the knot would be tightened creating a resistance against mechanical unfolding. Such an effect, if observable, should be contrasted with the result of "knot-free" stretching, that is, stretching the protein from N-terminus and from a position a few amino acid residues inside from the C-terminal. Stretching experiments as described above can be achieved by using the force mode of the atomic force microscope (AFM) which has been extensively used for imaging and manipulation of nanometer scaled objects including proteins and DNA since it was invented in 1986 [5]. To conduct a pulling experiment on a single protein molecule using AFM, we inserted cysteine residues next to C- and N-terminal residues when the protein was to be stretched with knot-tightening mode. For knot-free stretching, one of the cysteines was introduced next to N-terminal as above and the other at the 253rd position replacing the original glutamine residue by recombinant technology. We call the former "native" and the latter "E253C" according to the convention in molecular biology.

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<sup>1</sup>E-mail: ikai.a.aa@m.titech.ac.jp

## 2 Methods and materials

### 2.1 Preparation of proteins

The native and E253C proteins were prepared as described in [6] and kept at 4°C until used.

### 2.2 Atomic force microscopy

A Nanoscope III AFM (Digital Instruments, Santa Barbara, CA) was used in the force curve mode. Silicon nitride cantilevers with an integrated small stylus were used as AFM probes and crystalline silicon wafers cut into small pieces of approximately 1 cm square were used as the solid substrate. Details of the selection of cantilevers and the method of immobilization of protein samples to the AFM probe and the solid substrate are given in [6]. In the operation of AFM, a solid substrate with grafted proteins through covalent crosslinkers reactive to the -SH group of cysteine residues (SPDP (N-succinimidyl-3-[2-pyridyldithio]propionate; Pierce, Rockford, IL, USA) or NHS-PEG3400-MAL (Shearwater Polymers, Huntsville, AL, USA) as described in [7]) was immersed under a physiological buffer and an AFM probe that was modified with the -SH reactive crosslinker only was brought into contact with the proteins on the substrate. During a brief contact between the crosslinkers on the probe and the immobilized protein, a covalent bond was formed between them. Once the substrate started to retract from the probe, the protein molecule(s) sandwiched between the probe and the substrate was mechanically stretched until the covalent crosslinking system was broken by force. The AFM records the deflection of the cantilever ( $d$ ) at its free end and the distance covered by the substrate as driven by the piezo motor ( $D$ ). The extension of the protein sample ( $E$ ) is equal to  $(D-d)$  from which we constructed force-extension ( $F-E$ ) curves representing the relationship between the tensile force ( $F = k \times d$ ) and the extension of the protein ( $E$ ). Because of the large change of both the chain length and the cross-section of the protein during mechanical stretching, it was not possible to convert the relationship to a more universal one of stress-strain relationship.

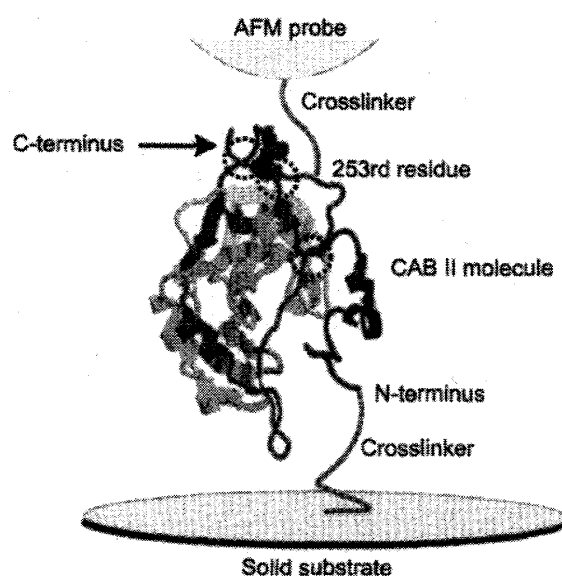


Figure 1: Schematic presentation of the experimental setup for the stretching experiment of protein molecules. The dotted circles show the three crossing points of the chain, together they form a pseudo knot conformation.

### 3 Results

The result of protein stretching experiments on the native CAB II had a force-extension feature as given in Figure 3 [8]. The theoretical contour length of the protein is about 91 nm assuming the average projected length of a single amino acid residue in an extended  $\beta$ -sheet conformation of a polypeptide to be 0.35 nm, but, with a force of several hundred of piconewtons, it is likely to be a little longer and close to 100 nm due to the forced opening of some of the bond angles. The native protein, however, was stretched up to 15 or 30 nm with a tensile force of less than 100 pN but whence the tensile force rapidly increased beyond 1 nN accompanied by little chain extension and the covalent bonds linking the probe to the substrate was ruptured consequently returning the cantilever to its unloaded position.

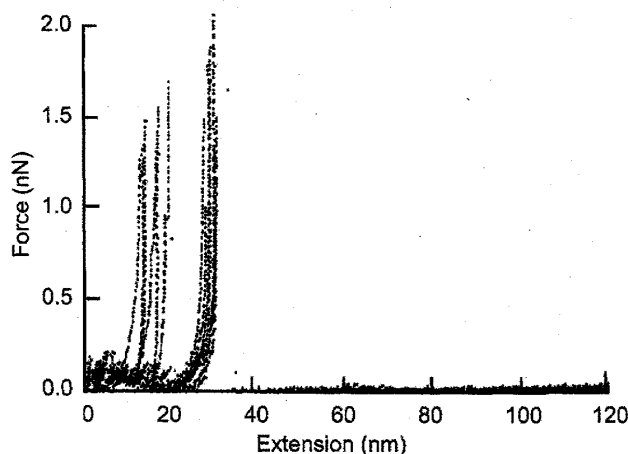


Figure 2: Representative  $F - E$  curves of the native form of CBA II.

In a remarkable contrast, the engineered protein, E253C, was extended up to 60 to 90 nm before the covalent system was ruptured with a force larger than 1.5 nN. The rather wide scattering of force curves was later ascribed to the presence of two conformational isomers of E253C which were produced in the process of protein expression in *E. coli*.

By using an affinity chromatography bearing a strong inhibitor for active CAB II, we were successful to separate two conformational isomers of E253C, one enzymatically active thus able to bind to the affinity chromatography and the other non-active. The former was called type 1 and the latter type 2. The difference between in type 1 and 2 was found in the degree of tertiary structure completion, namely, in type 1 both secondary and tertiary structures were complete with full enzymatic activity, whereas, in type 2, only the secondary structure was formed but no tertiary structure formation [6]. We measured  $F - E$  curves of type 1 and 2 conformers separately and the their presentive  $F - E$  curves are given in Figure 3.

Stretching of type 1 conformer was extended up to 60 - 70 nm with a concomitant non-linear increase of the tensile force and, when the tensile force reached 1.7 nN, the covalent structure was ruptured. The  $F - E$  curves of type 2 conformer revealed its softer nature compared with type 1 showing an almost full length stretching to about 90 - 100 nm before the covalent crosslinking system was broken at a tensile force of about 1.7 nN.

In Figure 3, the dotted lines represent occasional observation of the transition from type 1  $F - E$  curve to that of type 2 meaning that the breakdown of the tertiary structure in type 1 reduced it to type 2. Reversing the  $F - E$  curves from the extended to a shorter state did not show a reverse transition from type 2 to 1.

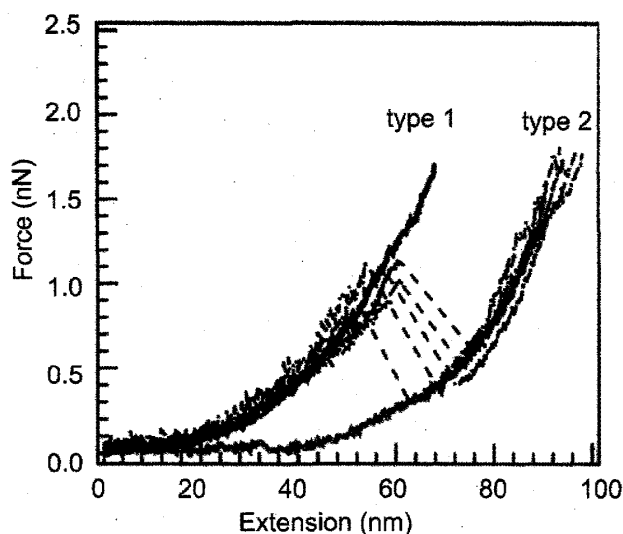


Figure 3: Representative  $F - E$  curves of type 1 and type 2 forms of E253C variants of CAB II.

## 4 Discussion

The mechanical resistance observed for the native conformation of CAB II at the extension length of 15 to 30 nm was most likely due to the knot tightening effect as we expected from its folding pattern. The resistance was, however, much greater than expected from what could be expected from the ordinary concept of the rigidity of non-covalently folded protein conformations which are roughly estimated to be disrupted by a force in the range of several tens to hundreds of pico newtons [9]. Another question was the presence of two types of force curves, one showing the steeply increasing resistance at around 15 nm of chain extension and the other around 30 nm. At this moment, we do not have a satisfactory explanation for this observation.

The  $F - E$  curves observed for the CAB II variant, E253C, were classified into two groups, namely, type 1 and 2. Type 1  $F - E$  curves represented extension of a fully folded conformation with an equivalent enzyme activity to the native one. Thus, if there were no knot tightening effect, the 3D conformation of CAB II could be extended up to 60-70 nm with a smooth but non-linear increase of the tensile force.  $F - E$  curves of type 2 variant revealed a much less rigid conformation compared with type 1. We investigated major differences between type 1 and 2 with respect to their conformations in a physiological buffer and found that the type 2 conformation was incomplete in the sense that it had almost all the secondary structures but lacked the final step of folding involving the knot completing process. In consequence, the enzymatic active site was not formed in type 2 and an essential  $\text{Zn}^{++}$  ion for the enzyme activity was not found associated with the protein. In another word, it had almost complete secondary structure but lacked tertiary structure. Occasionally there was a transition from type 1 stretching curve to that of type 2 but so far never from type 2 to 1.

Stretching of the secondary structure of CAB II to a fully extended conformation was accompanied by a dissipation of energy in the range of  $2 \times 10^4$  kJ/mol or  $3 \times 10^{-17}$  J/molecule. Destruction of the tertiary structure of CAB II was calculated by integrating the, thus, accompanied by a dissipation of energy in the order of  $6 \times 10^3$  kJ/mol, i.e.,  $1 \times 10^{-18}$  J/molecule.

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## 結び目構造を持つタンパク質の原子間力顕微鏡による延伸実験

猪飼 篤 東京工業大学イノベーション研究推進体、〒 226-8501 横浜市緑区長津田町  
4259 <sup>2</sup>

アブストラクト:炭酸デヒドラターゼは結び目構造を持つタンパク質としてよく知られており、そのN-末端とC-末端をナノ力学的方法により反対方向に引っ張ると伸張後の分子には三つ葉結び目 (trefoil knot) が形成される。単一タンパク質分子を引っ張るナノ力学実験は原子間力顕微鏡を用いて行うことができ、この実験の結果は結び目があるために両端を引っ張ると他の多くのタンパク質で見られるように分子構造が緩められるのではなく、反対に硬くなることが示された。すなわち、引っ張り実験が示す、タンパク質鎖の伸びと張力の関係を示すグラフはタンパク質分子がその全長である90–100 nmまで伸びる前に、およそ20 nm伸びた時点で大きな抵抗を受けることを示した。おなじタンパク質をC-末端ではなく、延伸する際に結び目を作らないように253番目の位置から引っ張ると分子は60–100 nmまで伸ばすことができたので、前述した20 nm付近における抵抗は結び目が絞られることに起因すると結論された。

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<sup>2</sup>E-mail: ikai.a.aa@m.titech.ac.jp